United States Department of Agriculture
Agricultural Marketing Service, Science & Technology
Microbiological Data Program

SOP No.: MDP-MTH-07		Page 1 of 12
Title: Detection of Pathogenic *E. coli* in Fresh Produce by Multiplex PCR (mPCR) and Cultural Isolation and Identification		
Revision: 02	Replaces: 09/01/05	Effective: 01/01/06

1. Purpose

To provide standard procedures for detecting Shiga toxin-producing *E. coli* (STEC) and Enterotoxigenic *E. coli* (ETEC) in preenriched cultures from produce wash samples. To isolate and identify these target organisms from positive samples.

2. Scope

This SOP shall be followed by all laboratories conducting microbiological studies for MDP, including support laboratories conducting non-routine activities that may impact the program. This SOP represents minimum MDP requirements and is presented as a general guideline. Each laboratory shall have written procedures that provide specific details concerning how the procedure has been implemented in that laboratory.

3. Principle

The presence of STEC and ETEC cells in the presumptive *E. coli* positive mixed cultures (from SOP MDP-MTH-01) will be detected using mPCR. This reaction will specifically amplify genes coding for Shiga toxins (Stx-1 and Stx-2) in STEC and the heat labile (LT-1) and heat stable (ST-1) toxins in ETEC.

4. Outline of Procedures

5. References

5.1. Florida Department of Agriculture and Consumer Services Protocol Number MTH-DEV-800, "Multiplex PCR detection of Shiga Toxin Producing and Enterotoxigenic *E. coli"*

5.2. SOP MDP-MTH-01, *Escherichia coli* MPN Method

5.3. SOP MDP-QA-03, Quality Assurance (QA) Controls

5.4. SOP MDP-SHIP-03, Procedures for Packaging, Shipping, and Archiving Microbiological Cultures

5.5. SOP MDP-DATA-01, Microbiological Record Keeping and Results Reporting

5.6. MPO Method Tryout, mPCR Screening for Virulent *E. coli*

5.7. Monday S. R., Shen Y., Keys C., Whittam T. S., and Feng P. 2005. Produce isolates of *Escherichia coli* Ont:H52 serotype that carry both Shiga toxin 1 and Stable toxin genes of *E. coli* (Manuscript submitted for publication)

6. Specific Procedures

6.1. Equipment and Materials

6.1.1. Sterile barrier pipette tips

6.1.2. PCR thermal cycler

6.1.3. Gel documentation system

6.1.4. Microcentrifuge

6.1.5. Electrophoresis apparatus

6.1.6. Power supply

6.1.7. VITEK®

6.1.8. Cooling block or ice bucket

6.1.9. PCR tubes

6.1.10. Incubator 35-37^{o}C

6.2. Media and Reagents

6.2.1. PCR grade water

6.2.2. Laboratory grade demineralized water (referred to as DI water)

6.2.3. 0.45 M Tris-Borate-0.01M EDTA (TBE) Buffer (Referred to as 5X TBE)

6.2.4. 100 base pair (bp) molecular size marker

6.2.5. 6X gel loading dye

6.2.6. Ethidium bromide 1% solution

6.2.7. 10X Primer Mix (Reference 5.7)

6.2.8. PCR Reagent Mix (Qiagen, Inc.)

6.2.9. NuSieve® Agarose 3:1 (Cambrex Bio Science Rockland Inc.)

6.2.10. CHROMagar™ *E. coli* (DRG International Inc.) or demonstrated equivalent

6.2.11. L-EMB agar

6.2.12. MacConkey agar (MA)

6.2.13. Xylose lysine deoxycholate agar (XLD)

6.2.14. Hektoen enteric agar (HE)

6.2.15. Blood agar plates (BA)

6.3. Controls:

6.3.1. Specific strains are listed in SOP MDP-QA-03.

6.3.2. The following controls shall be run with every batch of samples amplified as applicable. For both DNA and process controls, determine appropriate number of cells (CFU/mL) required for DNA extraction and PCR amplification in order to obtain clearly visible, well-separated DNA bands on agarose gels after electrophoresis. If any of the controls fail to yield a satisfactory result refer to SOP MDP-QA-03.

6.3.2.1. DNA controls: The DNA extraction and preparation of these controls should be made prior to sample setup. These controls shall be taken through the PCR amplification and electrophoresis steps.

6.3.2.1.1. Positive STEC DNA control

6.3.2.1.2. Positive ETEC DNA control

6.3.2.2. Process controls are taken through the DNA extraction, amplification, and electrophoresis steps along with samples.

6.3.2.2.1. Negative culture control (refer to SOP MDP-LABOP-02): Use DNA extracted from the negative control culture used in SOP MDP-MTH-04 and MDP-MTH-05.

6.3.2.2.2. Positive culture control (refer to SOP MDP-LABOP-02): Use DNA extracted from the positive control culture used in SOP MDP-MTH-05

6.3.2.3. Amplification control: Master mix with primer and PCR grade water (no DNA)

6.4. Safety

6.4.1. Wear UV protective goggles or face shield when working with a UV transilluminator that is not in a cabinet.

6.4.2. Wear gloves when preparing or handling the gel and staining solution. Ethidium bromide is a mutagen and a carcinogen.

6.5. Amplification

6.5.1. Use the extracted DNA obtained in SOP MDP-LABOP-02. For this procedure, test only the DNA from UPB preenriched cultures that tested positive for fluorescence in SOP MDP-MTH-01.

6.5.2. 1X Master Mix Preparation

6.5.2.1. Perform this procedure in a clean room or PCR workstation away from previously amplified material and general microbiological work area.

6.5.2.2. Label and assemble PCR tubes in cooling block or ice bucket.

6.5.2.3. Prepare 1X Master Mix by thawing 10X PCR primer mix, PCR grade water, and 2X mPCR master mix reagent. Invert tubes several times to mix after tubes are completely thawed. Centrifuge the tubes for 1-2 seconds. Prepare at least enough master mix for (n + 1) reactions, (where n equals the number samples), according to the following table.

Preparation of 1X Master Mix			
Reagent	**1 Reaction**	**(n) + 1 Reactions**	**Final Concentration**
2X PCR Master Mix	25 µL	25(n) + 25 µL	1X
10X PCR primer	5 µL	5(n) + 5 µL	1X
PCR grade water	15 µL	15(n) + 15 µL	---
Total volume	45 µL	45(n) + 45 µL	---

6.5.3. Aliquot 45 µL of the 1X master mix into each PCR tube.

6.5.4. Follow the manufacturer's directions for storage of reagents.

6.5.5. Add 5 µL of DNA sample to the PCR tubes prior to amplification. Add 5 µL of PCR grade water to the tube that serves as the amplification negative control. This tube does not receive the 5 µL of sample DNA.

6.5.6. Multiplex PCR Thermal Cycler Parameters

Component	Parameters	Number of Cycles
Denaturation and activation of HotStart® Taq polymerase	95ºC for 15 minutes	1 cycle
Touch down program	95ºC for 30 seconds	10 cycles
	69ºC to 60ºC (reduce 1ºC per cycle) for 20 seconds	
	72ºC for 30 seconds	
Conventional PCR program	95ºC for 30 seconds	30 cycles
	60ºC for 20 seconds	
	72ºC for 30 seconds	
Extension step	72ºC for 7 minutes	1 cycle
Soak step	4ºC indefinitely	1 cycle

6.5.7. Storage of Samples

DNA extracts and PCR-amplified samples may be maintained at 2-8ºC for short-term storage or -20º for long-term storage. Store amplified samples away from unamplified material. Place samples in a cooling block if using a frost-free freezer to prevent freezing/thawing of samples.

6.6. Detection

6.6.1. Gel Preparation

6.6.1.1. Prepare a 1:10 dilution of 5X TBE buffer in DI water to obtain a working solution of 0.5X TBE.

6.6.1.2. Prepare a 1.5 % (w/v) agarose gel using 0.5X TBE, swirl to mix.

6.6.1.3. Heat until the agarose is completely dissolved in the buffer. Let cool slightly on countertop.

6.6.1.4. Pour agarose onto a clean, dry gel platform containing a comb at one end.

6.6.1.5. After the gel has completely solidified, place it and the platform into the electrophoresis chamber with the well end towards the negative electrode.

6.6.1.6. Add sufficient 0.5X TBE to the chamber to cover the gel.

6.6.1.7. Remove the comb and ensure that the wells are filled with 0.5 X TBE buffer.

6.6.2. Sample Preparation and Electrophoresis

6.6.2.1. Pipette 2 µL of 6X loading solution for each sample into a 1.5 mL tube.

6.6.2.2. Add 10 µL of sample to each 2 µL drop of loading solution and centrifuge for 1-2 seconds.

Note: Alternatively, sample may be added to loading solution on parafilm.

6.6.2.3. Add 5µL of 100 bp molecular weight marker to 1µL of 6X loading solution or based on manufacturer's suggestion or empirical data.

6.6.2.4. Transfer the samples and solution with molecular weight marker to corresponding wells in the gel.

6.6.2.5. Place the cover on the electrophoresis chamber, and connect the leads so that the DNA migrates towards the positive electrode.

6.6.2.6. Run the gel electrophoresis at 100 Volts. Monitor the blue dye migration to estimate band separation for distinct separate visible bands when exposed to UV light ~3/4ths the length of the gel.

6.6.2.7. Turn off power and remove cover of electrophoresis chamber.

6.6.3. Staining of Gel

6.6.3.1. Ethidium bromide solution: add 40 µL of 1% ethidium bromide into 400 mL of 0.5X TBE or DI water to a final concentration of 1µg/mL.

6.6.3.2. Place staining solution and gel in a polypropylene plastic box which is either opaque or covered with aluminum foil. Stain gel approximately 20 minutes in the dark, with occasional, gentle swirling of the staining solution.

6.6.3.3. Remove gel from staining solution and rinse with DI water.

Note: The staining solution may be used to stain multiple gels. Store in the dark. Discard staining solution by appropriate means.

6.6.4. Gel Documentation

6.6.4.1. Place gel along with tray on gel documentation system or a UV (ultraviolet) transilluminator.

6.6.4.2. Expose gel to UV light.

6.6.5. Obtain image of gel.

6.7. Identification

6.7.1. Identify the 200 bp fragment of the molecular weight marker lanes closest to each sample.

6.7.2. The 16S rDNA internal control band should be visible at 206 bp in each sample lane and in the negative culture control lane. No bands should be detected in the amplification control lane. The 16S rDNA band (206-bp) may or may not be visible in the positive control lanes as well as samples that tested positive.

6.7.3. Compare the results of the sample lanes to the following table for STEC and ETEC identification

Control Strains	Toxin	Base Pair Fragment Sizes
STEC	Stx 1 and Stx 2	313 bp
ETEC	LT-1	416 bp
	ST-1	169 bp

6.8. Isolation

Note: Laboratories are to use professional skill and judgement in isolating pathogenic *E. coli*. MPO encourages laboratories to use additional methods and techniques such as incubating plates at 42°C or using various other selective media. MPO strongly encourages laboratories to run the mPCR and begin isolation procedures on the fresh cultures and not to freeze them. The mPCR technique may detect other bacteria that can harbor toxin genes found in other bacteria (e.g. *Shigella dysenteriae* which contains a shiga toxin 1 gene identical to STEC). Notify MPO even if the final VITEK® result is not an *E. coli* and document the procedure used.

6.8.1. For each mPCR-positive sample, prepare five CHROMagar™ E. coli and five L-EMB plates. MacConkey agar plates can also be used. The Stx-positive cultures may contain *Shigella dysenteriae* type 1 and use of other selective plates such as HE and XLD (five plates total) should be considered. Streak and/or plate (0.1 mL or diluted) the UPB preenrichment culture that tested positive for mPCR on the selective agar plates for isolation. Incubate at 35-37ºC for 18-24 hours.

6.8.2. Examine the plates for typical *E. coli* colonies. Pick a minimum of 20 typical colonies (if available) from the selective agar plates.

Typical colony characteristics of pathogenic *E. coli*		**Other Organisms of Interest**
Medium/Test	**Colony Characteristics**	
MA	Red to pink	
L-EMB	Blue-black and green w/metallic sheen	
XLD	Yellow	*Shigella* - red
HE	Salmon-orange	*Shigella*- greenish blue
CHROMagar® *E. coli*	Blue	*E. coli* O157:H7 - white

6.8.2.1. Refer to SOP MDP-QA-03 Attachment 1, Current QA Control Strain Information, for characteristics of control strains.

6.8.3. Inoculate each typical colony into 2 to 10 mL single-strength LST broth. Incubate overnight at 35-37°C.

6.8.4. Mix the broth cultures. Up to 10 broths may be pooled by adding 10 µL from each broth into a sterile tube. Run samples through DNA extraction, mPCR and gel electrophoresis, and gel imaging.

6.8.5. Individual LST broth cultures must be run when a positive detection is made on a pooled sample.

6.8.6. Identify the mPCR positive culture. Streak and/or plate (0.1 mL or can be diluted) the culture on the selective agar plates for isolation. Incubate at 35-37°C for 18-24 hours.

6.8.7. Pick 3-5 individual typical colonies and restreak on BA, CHROMagar *E. coli* and L-EMB or MacConkey plates. Use the culture from BA plates and run on VITEK® to identify the isolate is an *E. coli*.

6.8.8. Repeat mPCR on 3 isolates. Choose one isolate that is identified as E. coli and carries toxin gene (s) for archiving and shipping.

6.8.9. Select one typical colony that has been confirmed by mPCR for archiving and shipping according to SOP MDP-SHIP-03.

6.9. Reporting

6.9.1. A final positive result is defined as an isolated organism that produces the 169, 313, or 416 bp band alone or in combination.

6.9.2. Data shall be reported according to SOP MDP-DATA-01 with the following exceptions:

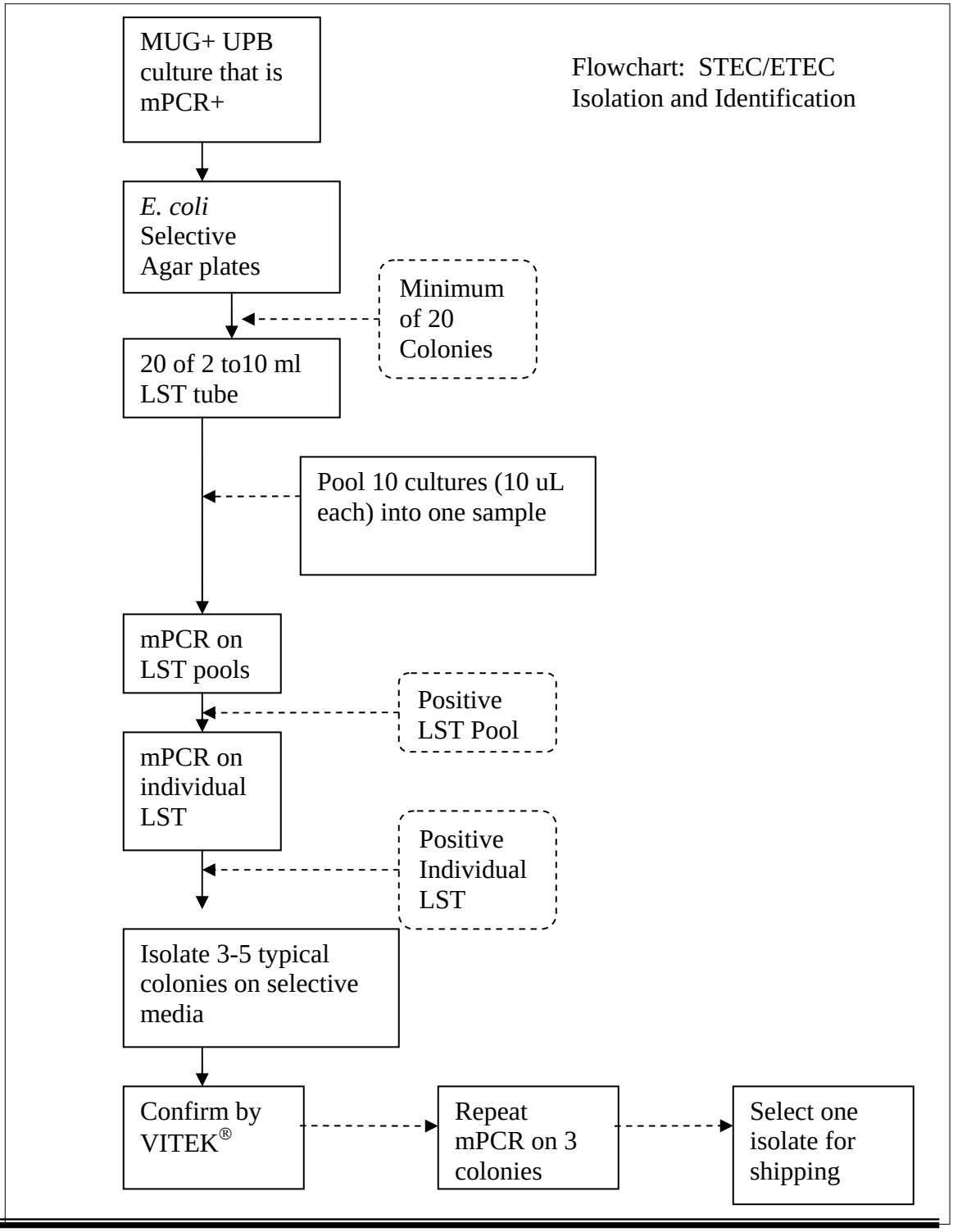
6.9.2.1. Preliminary positive and final results for this procedure do not need to be reported to MPO using the SOP MDP-DATA-01 Attachment 01, Preliminary / Final Results Notification Form.

6.9.2.2. However, if VITEK® identifies the organism as a *Shigella* sp. or *E. coli* O157, MPO shall be notified using the attachment mentioned above.

6.10. Parameters

Parameters used for individual laboratory validations of this procedure are on file at MPO. Laboratories proposing a change to existing parameters shall contact MPO to determine extent of validation requirements.

Disclaimer: Reference to brand names (kits, equipment, media, reagents, etc.) does not constitute endorsement by this agency.


Flowchart: STEC/ETEC Isolation and Identification
MUG+ UPB culture that is mPCR+
E. coli Selective Agar plates
Minimum of 20 Colonies
20 of 2 to10 ml LST tube
Pool 10 cultures (10 uL each) into one sample
mPCR on LST pools
Positive LST Pool
mPCR on individual LST
Positive Individual LST
Isolate 3-5 typical colonies on selective media
Confirm by VITEK®
Repeat mPCR on 3 colonies
Select one isolate for shipping

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Revision 02 January 2006 Monitoring Programs Office

- Changed test sample to extracted DNA from UPB preenriched culture (cultures of positive samples from SOP MDP-MTH-01).
- Positive produce control (SOP MDP-LABOP-02) replaces ETEC process control
- Expanded isolation step

Revision 01 June 2005 Monitoring Programs Office

- Allowed use of loop in 6.9.2.
- Storage –adjusted text to ensure minimize freezing and thawing of vials
- Removed DNA control using mix of STEC:ETEC at 1:1
- Deleted Attachment 01, mPCR Validation Protocol to allow laboratories to contact MPO to determine validation requirements for proposed changes to parameters
- Clarified reporting requirements

Original March 2005 Monitoring Programs Office

- Established procedures and requirements for mPCR screening for virulent *E. coli*